AD_____

Award Number: W81XWH-10-1-0051

TITLE: Hsf1 in Her2-positive Breast Cancer

PRINCIPAL INVESTIGATOR: Michael Sherman, Ph.D.

CONTRACTING ORGANIZATION: Boston University Medical Campus
Boston, MA 02118-2436

REPORT DATE: February 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
February 2011	Annual	15 January 2010 – 14 January 2011

4. TITLE AND SUBTITLE

Hsf1 in Her2-positive Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0051

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Michael Sherman

E-Mail: sherma1@bu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Boston University Medical Campus
Boston, MA 02118-2436

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the first year of the grant we addressed tasks 1, 2 and 3. Specifically we demonstrated that Hsf1 controls development of Her2-positive cancer both at the stage of initiation, where is affects the oncogene-induced senescence, and later at the stage of progression, where it affects tumor angiogenesis. We also dissected the mechanism of effects of Hsf1 on tumor angiogenesis, and demonstrated that Hsf1 regulates expression of the major angiogenesis factor HIF-1.

15. SUBJECT TERMS
Hsf1, Her2 positive cancer, angiogenesis, Hif1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Recently it was demonstrated that tumorigenesis in several models strictly requires the heat shock transcription factor Hsf1. Indeed crossing of *p53*$^{-/-}$ mice with *hsf*$^{-/-}$ mice almost completely prevented lymphoma development [1], but not appearance of some other cancers. Similarly, Hsf1 deficiency drastically delayed chemical skin carcinogenesis and increased survival from 30% to 90% [2]. In this study, using transgenic and xenograft models, we uncovered that Hsf1 is critical for development of Her2-positive breast cancer. Preliminary data suggested that Hsf1 is necessary for suppression of the oncogene-induced senescence (OIS), and for tumor angiogenesis. The major goal of this program was to investigate the molecular nature of these effects.

Body

Task 1

Hsf1 knockout suppresses Her2-induced hyperplasia and tumor development

We have previously found that the knockdown of Hsf1 in MCF10A human mammary epithelial cells prevents neoplastic transformation by Her2 oncogene. Indeed, while expression of Her2 in control MCF10A cells facilitated foci formation in culture and tumor appearance in nude mice, expression of this oncogene in Hsf1 knockdown MCF10A cells led to growth arrest and OIS, and tumors could not form in nude mice [3]. To further dissect where in the tumorigenic process Hsf1 exerts its activity, here we used the transgenic animal model. We crossed Hsf1 knockout animals with mice expressing Her2/NeuT (a rodent homolog of Her2 carrying activating mutation) under the control of MMTV promoter (MMTVneu) [4] to generate WT-MMTVneu^{+}, hsf1$^{+/-}$MMTVneu^{+}, and hsf1$^{-/-}$MMTVneu^{+} mice.

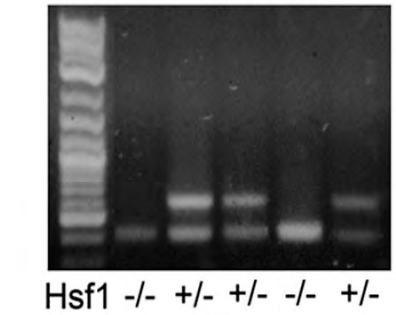


Fig. 1. Lack of Hsf1 in the knockout animals is shown by PCR.

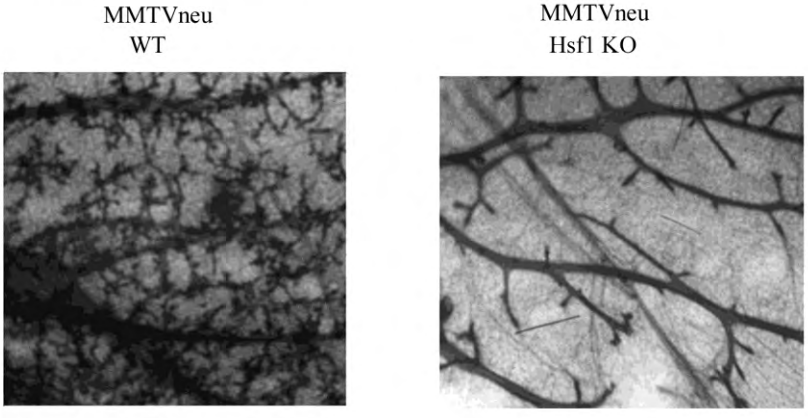


Fig. 2 Knockout of Hsf1 blocks NeuT-induced mammary duct and alveoli branching. WT-MMTVneu^{+} and hsf1$^{-/-}$MMTVneu^{+} mice were sacrificed at 3-months of age and their mammary gland whole mounts were observed after Carnoy's fixative and staining with carmine.

To investigate the role of Hsf1 in Her2-induced hyperplasia, mammary glands were taken from 3-month old virgin mice to evaluate duct branching. Expression of Her2 in WT-MMTVneu^{+} mammary gland led to high density of ducts and extensive alveoli branching, as reported previously [5]. Importantly, hsf1$^{-/-}$MMTVneu^{+} animals there was a low duct density, and almost no alveoli branching (Fig. 2). Therefore, Hsf1 KO prevented Her2-induced tissue hyperplasia, possibly by aggravating senescence, similar to what we have found recently with NeuT-induced mammary tumors in the Hsp72 knockout mouse model [6].

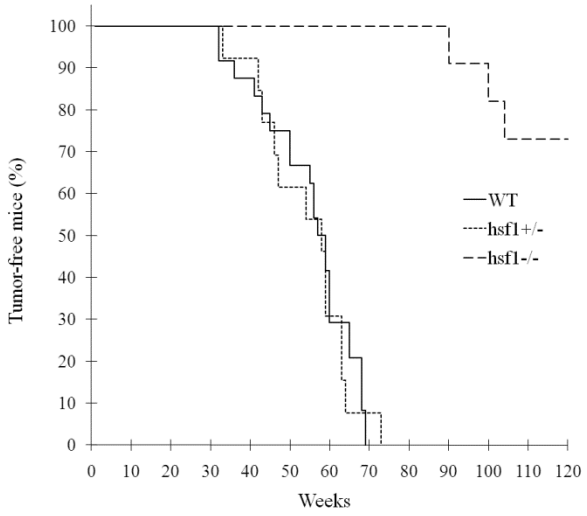


Fig. 3 Emergence of NeuT-induced tumors in WT-MMTVneu^{+} (n=16), heterozygotes hsf1$^{+/-}$MMTVneu^{+} (n=13), and hsf1$^{-/-}$MMTVneu^{+} (n=11) animals.

To address whether Hsf1 KO suppresses NeuT-dependent tumorigenesis *in vivo,* we analyzed effects of hsf1 knockout on NeuT-induced tumor development. There was similar tumor incidence between heterozygous hsf1$^{+/-}$MMTVneu^{+} and WT-MMTVneu^{+} mice (median tumor appearance in this strain was about 55 weeks), indicating that one copy of the Hsf1 gene is sufficient to support mammary tumor emergence induced by Her2/NeuT (Fig. 3). In contrast, the absence of Hsf1 in homozygous knockout animals markedly inhibited mammary tumor development (Fig. 3). Indeed, tumors emerged with strong delay, and only three tumors of eleven animals appeared. Therefore, this model of Her2-positive breast cancer establishes that Hsf1 is critical for tumor initiation and hyperplasia.

Task 2

Hsf1 knockdown suppresses tumor growth and angiogenesis

To investigate whether Hsf1 has additional effects on later stages of tumor development, we measured growth rates of rare tumors that emerge in hsf1 KO animals compared to control mice. Indeed, these tumors grew significantly slower than in control animals (Fig. 4) indicating that Hsf1 may be required not only for NeuT-induced initial transformation, but for tumor progression as well.

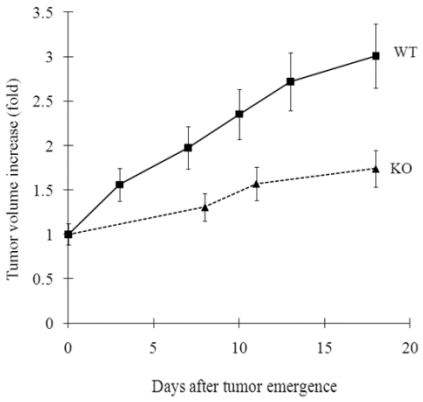


Fig. 4 NeuT-induced tumors in Hsf1 KO mice demonstrate reduce growth rate. The data shown are means +/-SEM.

Since among major factors limiting growth of solid tumors *in vivo* is neovascularization, we excised tumors from control and knockout animals, prepared slides and immunostained them with a marker of

angiogenesis (endothelial cells) CD31. We observed that although the number of blood vessels was similar in wt and k/o animals (not shown), the mean vessel area in tumors from Hsf1 knockout animals was almost twice as low as in wild type animals (Fig. 5,6), indicating that the vessels were underdeveloped.

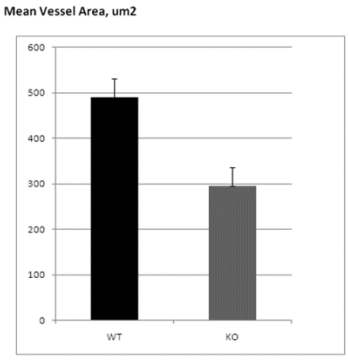


Fig. 5 Tumors in Hsf1 KO mice demonstrate reduced angiogenesis. Tumors from WT and KO animals were excised, fixed, stained for endothelial marker CD31, and analyzed for mean vessel area.

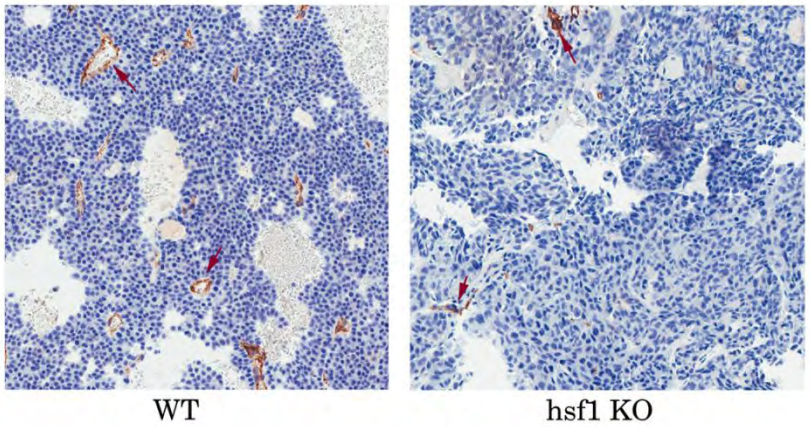


Fig. 6 Tumor tissue staining with anti-CD31 antibody. Tumors from control and Hsf1 KO animals are shown.

Task 3

Hsf1 knockdown suppresses tumor growth and angiogenesis in xenograft model

Since in transgenic mouse model Hsf1 is lacking both in mammary tumor and surrounding stroma, to understand mechanisms by which Hsf1 can regulate angiogenesis we decided to switch from NeuT-induced mouse mammary tumors to a simpler system, i.e. xenograft with human breast cancer cells following Hsf1 knockdown. In this system Hsf1 can be downregulated specifically in human tumor cells but remain expressed normally in surrounding mouse stroma. As we reported previously, growth of many cancer cell lines (e.g. NeuT-expressing MCF10A cells or MDA-MB453) is dependent on Hsf1, since Hsf1 knockdown causes senescence due to accumulation of p21 and downregulation of the mitotic and anti-apoptotic protein survivin [3]. Therefore these cell lines cannot propagate even in vitro upon depletion of Hsf1, and, accordingly, effects of Hsf1 on angiogenesis cannot be studied in this system.

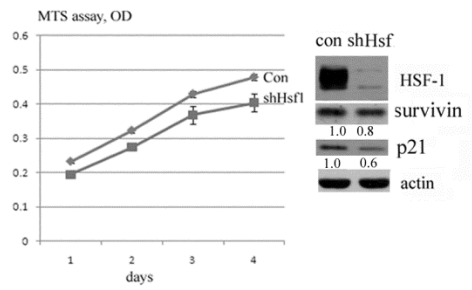


Fig. 7 Knockdown of Hsf1 has a little effect on growth of MCF7 cells in vitro and it does not increase p21 or decrease survivin levels. Cells were infected with shHsf1 retrovirus and selected for 4 days; their growth was assessed by MTS assay, and expression of p21 and survivin by immunoblotting.

To avoid this problem, we screened several breast tumor cell lines and found that Hsf1 knockdown does not decrease survivin levels and does not increase p21 levels in MCF7 human breast carcinoma (Fig. 7). Accordingly, growth of these cells in vitro was not significantly affected by Hsf1 knockdown (Fig. 7).

Therefore, we have chosen MCF7 cells to assess effect of Hsf1 knockdown on tumor angiogenesis and growth *in vivo* in the xenograft model. MCF7 cells were infected with retroviral vector expressing shHsf1 as described before and selected with puromycin for 5 days. To avoid possible variations of host factor(s) which could affect tumor growth, control cells were injected in right flanks and shHsf1 knockdown cells – in left flanks of the same animals and their growth was monitored by caliper. Tumors emerged at the sites of injection of both control and Hsf1-depleted cells on day 9 after inoculation (Fig. 8). Importantly, after tumor emergence, tumors formed by control MCF7 cells grew rapidly, while tumors formed by the Hsf1 knockdown ceased to grow soon after emergence (Fig. 8). The strong inhibitory effect of Hsf1 knockdown on growth of MCF7 cells in xenografts was in sharp contrast with cell culture, where Hsf1 knockdown practically did not affect the growth rate (Fig. 7). We have isolated tumors, stained them for CD31 as described above, and found that, similar to NeuT-induced mammary tumors in Hsf1 KO mice, Hsf1 knockdown markedly decreased mean vessel area in MCF7 human breast cancer xenografts (Fig. 9) (of note, there was no significant difference in the number of vessels). These data indicate that the xenograph model recapitulates effects of Hsf1 seen in transgenic model, and suggest that control of tumor angiogenesis may represent an important factor regulated by Hsf1.

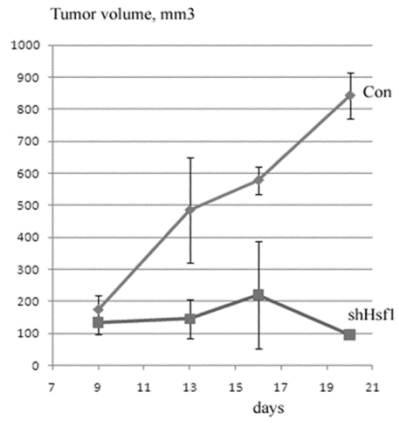


Fig. 8 Knockdown of Hsf1 blocks growth of MCF7 cells *in vivo* in xenografts. Cells infected with shHsf1 retrovirus as in A were injected in nude mice (10^6 cells per injection) and growth of tumors was monitored by caliper.

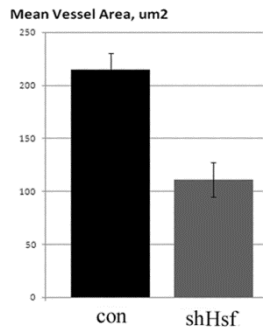


Fig. 9 Xenograft tumors from animals (as described in Fig. 8) were excised, fixed, stained for CD31 and analyzed for mean vessel area as in Fig. 6. Data are means+/-SE.

Hsf1 controls expression of the hypoxia-inducible factor HIF-1

Hypoxia-inducible factor 1 HIF-1 is considered to be the major regulator of tumor angiogenesis [7, 8], and therefore we assessed its expression in xenografts formed by MCF7 cells with Hsf1 knockdown. We found high levels of HIF-1 in control tumors (which indicated hypoxic conditions in xenografts) but in tumors with Hsf1 knockdown there were much lower levels of HIF-1 (Fig. 10). Similarly, in Hsf1 knockdown tumors we observed downregulation of HIF-1 target CAIX (carbonic anhydrase 9) (Fig. 10).

Accordingly, Hsf1 appears to control angiogenesis in xenografts via regulating accumulation of HIF-1□.

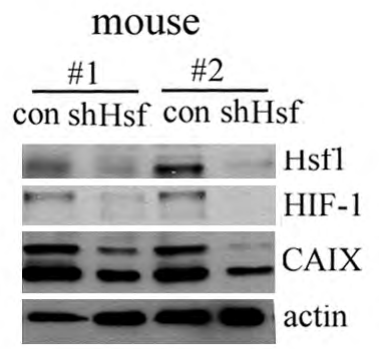


Fig. 10 Knockdown of Hsf1 reduces expression of HIF-1 and its target CAIX in xenografts. Expression of HIF-1 and CAIX tumor xenografts was analyzed for by immunoblotting.

To elucidate mechanisms by which Hsf1 regulates HIF-1□ expression, we studied effects of Hsf1 knockdown on HIF-1 expression in cell culture. Control and shHsf1 MCF7 cells were exposed to hypoxia (1 % oxygen for 16 hours) or hypoxia mimetic DFO (100 μM), and levels of HIF-1□ were monitored by immunoblotting. Knockdown of Hsf1 markedly suppressed accumulation of HIF-1 in response to these stimuli (Fig. 11), similar to suppression of HIF-1accumulation in xenografts formed by shHsf1 MCF7 cells (Fig. 10). Importantly, Hsf1 knockdown also strongly inhibited secretion of VEGFA, the major growth factor responsible for neovascularization, as well as other targets of HIF-1□□□CAIX and Glut-1 (Fig. 12, 13).

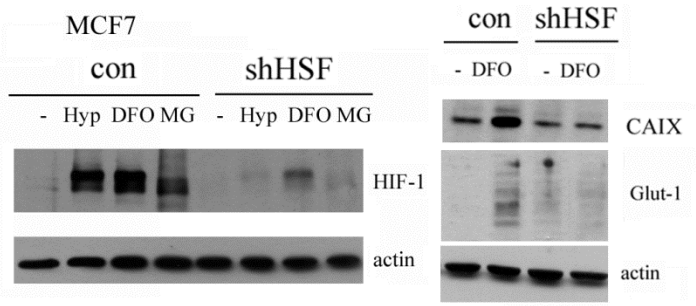
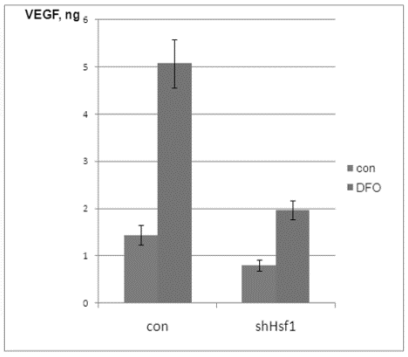


Fig. 11 Knockdown of Hsf1 in MCF7 cells inhibits HIF-1 accumulation after hypoxia (hyp, 1% 0_2, 16hr), hypoxia mimetic deferoxamine (100 μM, 4 hr), or proteasome inhibitor MG132 (5 μM, 4 hr). Cells were infected with shHsf1 retrovirus, and HIF-1 expression was analyzed by immunoblotting. Hsf1 knockdown also reduces induction of HIF-1a targets CAIX and Glut-1 in MCF7 cells.



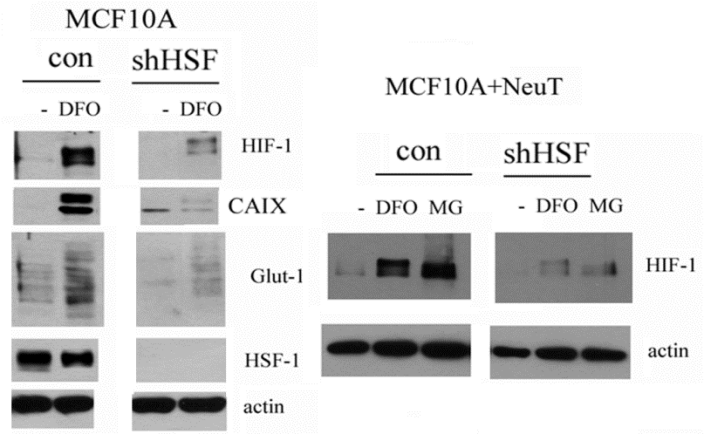


Fig. 12 Hsf1 knockdown reduce induction of HIF-1a target VEGF in MCF7 cells. Cells were infected with Hsf1 retrovirus, treated with DFO for 48 hr, and medium was collected and analyzed by ELISA for VEGFA by Quansys Biosciences.

Fig. 13 Knockdown of Hsf1 inhibits accumulation of HIF-1 and its targets in MCF10A (left panel) and NeuT-infected MCF10A cells (right panel). Cells were treated with DFO (100 μM, 4

hr) or MG132 (5 μM, 4 hr) and accumulation of HIF-1 and its targets CAIX and Glut-1 was analyzed by immunoblotting.

To assess whether effect of Hsf1 knockdown on HIF-1□ has a general significance, we used other breast cell lines, including normal untransformed cells MCF10A, NeuT-transformed MCF10A, Her2-positive cancer lines MB453 and BT474, and triple-negative Hs578T. In all these cell lines Hsf1 knockdown strongly inhibited accumulation of HIF-1 and its targets CAIX and Glut1 in response to hypoxia mimetic DFO (Fig. 14).

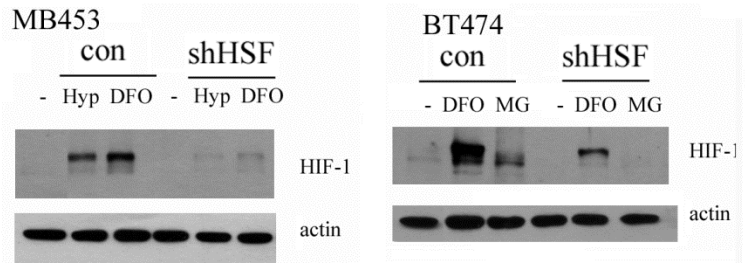


Fig. 14 Her2-positive MB453 (left panel) and BT474 cells (right panel) were infected with Hsf1 retrovirus as in A, treated with hypoxia for 16 hr, or DFO (100 μM, 4 hr), or MG132 (5 μM, 4 hr) and accumulation of HIF-1 was analyzed by immunoblotting.

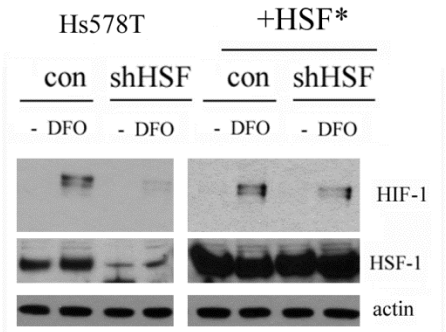


Fig. 15 Expression of the shRNA-resistant Hsf1 mutant, reverses the effect of shRNA on HIF-1α. Hs578T cells were infected with retrovirus encoding Hsf1* or control retrovirus, and selected. Then Hsf1 was depleted by shRNA, and HIF-1α expression was measured by immunoblotting in naïve cells or following DFO treatments.

This phenomenon was not an off target effect of Hsf1 knockdown, since expression of shRNA-resistant mutant of Hsf1 (Hsf1*) prevented downregulation of HIF-1□□□Therefore, impairment of angiogenesis in NeuT-induced mammary tumors in *hsf1* knockout animals can be associated with inhibition of HIF-1□□expression in tumors. Of note, these effects were not limited to Her2-positive breast cancer cells, since they were seen in Her2-negative, ER positive MCF7 cells and in triple-negative cancer line Hs578T (Fig.15).

Key Research Accomplishments:

- Demonstrated that Hsf1 knockout inhibits Her2/NeuT-induced hyperplasia of mammary tissue.
- Demonstrated that Hsf1 knockout inhibits Her2/NeuT-induced tumor emergence.
- Demonstrated that Hsf1 knockout inhibits growth of Her2/NeuT-induced tumors.
- Demonstrated that Hsf1 knockout inhibits angiogenesis in Her2/NeuT-induced tumors.
- Developed a xenograft model that recapitulates effects of hsf1 on tumor growth.
- Demonstrated that in this xenograph model Hsf1 controls angiogenesis.
- Demonstrated that Hsf1 controls the angiogenesis transcription factor HIF-1 and its targets in xenografts and culture of breast cancer cells.

Reportable outcomes:

Manuscript in preparation

Conclusion:

This study demonstrates that Hsf1 controls development of Her2-positive cancer both at the stage of initiation, where is affects the oncogene-induced senescence, and later, where it affects tumor angiogenesis. It also dissects the mechanism of effects of Hsf1 on tumor angiogenesis, and demonstrates that Hsf1 regulates expression of the major angiogenesis factor HIF-1. This program provides rationale for development of Hsf1 inhibitors that can inhibit multiple pathways in tumor initiation and progression. It also establishes a simple xenograft model that can be used for anti-Hsf1drug testing.

References

1. Min JN, Huang L, Zimonjic DB, Moskophidis D, Mivechi NF. Selective suppression of lymphomas by functional loss of Hsf1 in a p53-deficient mouse model for spontaneous tumors. Oncogene. 2007 Aug 2;26(35):5086-97.
2. Dai C, Whitesell L, Rogers AB, Lindquist S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell. 2007 2007 Sep 21;130(6):1005-18.
3. Meng L, Gabai VL, Sherman MY. Heat-shock transcription factor HSF1 has a critical role in human epidermal growth factor receptor-2-induced cellular transformation and tumorigenesis. Oncogene. 2010 Sep 16;29(37):5204-13.
4. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A. 1992 Nov 15;89(22):10578-82.
5. Muller WJ, Arteaga CL, Muthuswamy SK, Siegel PM, Webster MA, Cardiff RD, et al. Synergistic interaction of the Neu proto-oncogene product and transforming growth factor alpha in the mammary epithelium of transgenic mice. Mol Cell Biol. 1996 Oct;16(10):5726-36.
6. Meng L, Hunt C, Yaglom JA, Gabai VL, Sherman MY. Heat shock protein Hsp72 plays an essential role in Her2-induced mammary tumorigenesis. Oncogene. 2011 Feb 7;30(25):2836-45.
7. Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev. 2007 Feb;17(1):71-7.
8. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene. 2009;29(5):625-34.